EFFECT OF α-AMANITIN ON CHROMOSOMAL AND NUCLEOLAR RNA-SYNTHESIS IN *CHIRONOMUS THUMMI* POLYTENE CHROMOSOMES

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1. Introduction

Recently it was established [1–3] that the toxic octapeptide α-amanitin inhibits the ^{3}H-uridine labelling in polytene chromosomes whereas incorporation into the nucleolus remains unaffected. This result is in good agreement with data from *in vitro* experiments indicating that α-amanitin inhibits only the extranucleolar RNA polymerase form II but not the nucleolar form I [4–6] and the second known extranucleolar form III [4].

In the present paper evidence is presented that in explanted salivary glands of *Chironomus thummi* larvae α-amanitin exclusively prevents the synthesis of high molecular weight RNA (H RNA) localized in chromosomal puffs. The synthesis of low molecular weight (4–5 S) chromosomal RNA and nucleolar pre-ribosomal RNA remains uninhibited. The processing of the 38 S rRNA precursor, however, is delayed.

2. Material and methods

Salivary glands of 4th instar larvae of *Ch. thummi* were incubated in 20 μl Cannon's modified insect medium [7] containing 100 μCi ^{3}H-uridine, 100 μCi ^{3}H-cytidine (24.5 Ci/mmole and 18.8 Ci/mmole, respectively, UVVVR, Prague, Czechoslovakia) and/or 1 μg/ml α-amanitin (for autoradiography 0.5 μg/ml). After incubation, the glands were fixed [8] and single genome parts isolated at room temp by means of a sliding micromanipulator (Carl Zeiss, Jena). RNA was isolated by pronase digestion [8] and precipitated in the presence of 30–50 μg cold *Ch. thummi* RNA obtained by phenol extraction [9]. RNA was analysed on gel columns [10] consisting of 2 parts, with 2.4% (4.5 cm) and 7.2% (3 cm) acrylamide, respectively. After a 30 min prerun at 5 mA/tube samples were applied to the gels in 30–50 μl electrophoresis buffer containing 10% sucrose (w/v) and run at 3.5 mA/tube and 4° for 2.5–3 hr. After fixation, staining by methylene blue and destaining [11] gels were scanned at 595 nm in a Joyce-Loebl-chromoscan densitometer. For measuring the ^{3}H-radioactivity gels were frozen, cut into 1 mm slices, each slice dissolved in 0.2 ml H_2O_2 (1–5 hr at 55°) and, after adding 10 ml Bray's solution, counted in a Tricarb scintillation counter at a background of 30 cpm. Quench correction was made for each sample and the radioactivity calculated as disintegrations per 10 min.

Autoradiographs were prepared from whole glands or from some cells of glands used for RNA extraction.

3. Results

As described previously [2] and further confirmed in additional autoradiographic experiments, α-amanitin at a concentration of 0.5 μg/ml inhibits the labelling of puffs and Balbiani rings in *Ch. thummi* salivary glands explanted in ^{3}H-uridine supplemented medium. The labelling of nucleoli remains unaffected whereas the diffuse label of the chromosomes is increased. The

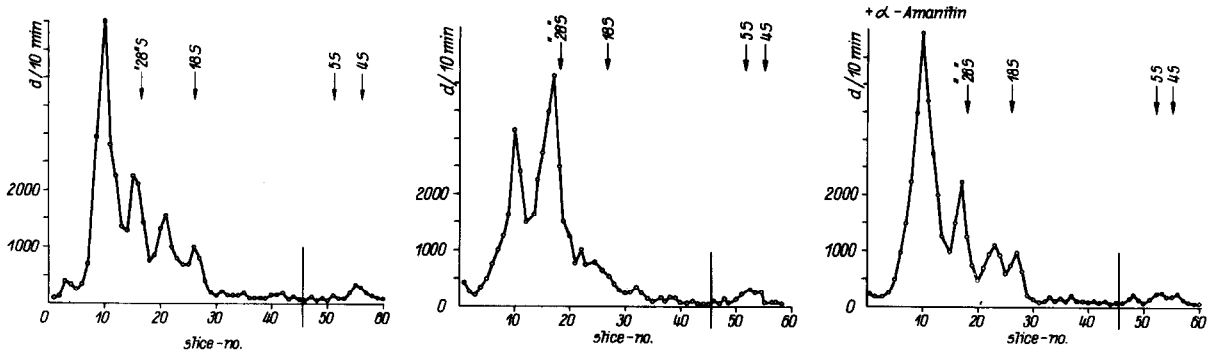

Fig. 1. Labelling of nucleolar RNA in the absence (a, b) and presence (c) of α-amanitin. 4 salivary glands of *Ch. thummi* were pre-incubated in Cannon's modified medium containing 1 μg/ml α-amanitin followed by a 4 hr incubation in the same medium supplemented with ^{3}H-uridine and ^{3}H-cytidine. As one control the 4 sister glands were incubated 4 hr under the same conditions in α-amanitin-free medium (b). In another control experiment, 4 glands were incubated in α-amanitin-free medium for only 1 hr (a). After incubation glands were fixed, nucleoli (30–50) isolated and RNA extracted and separated in polyacrylamide gels. The border between the 2 parts of the gel is marked by a vertical line on the abcissa. For further details see Materials and methods.

reaction was complete in all experiments insofar as all cells of a given gland reacted. In 40–50% of the autoradiographs, however, a small number of puffs on chromosomes I–III showed distinct label. We were unable to localize these regions exactly on the chromosome map, but apparently not always the same chromosomal regions remained unaffected in different glands or even cells of the same gland, as demonstrated after DRB-treatment of *Ch. tentans* salivary glands [12]. Treatment up to 48 hr of whole larvae in water containing up to 10 μg/ml α-amanitin was without any effect on the labelling pattern of the chromosomes. After injection of α-amanitin into larvae together with ^{3}H-uridine (0.5 μCi/animal) or 30 min before, the incorporation of radioactivity into the salivary gland cells (but not into the cells of the glandular duct) was too low in control and experimental animals to demonstrate any clear-cut effects.

The electrophoretic analysis of RNA extracted from nucleoli of untreated glands revealed after 1 hr incubation, besides a main 38 S* fraction, 3 peaks at the 30 S, 23 S and 18 S position and a small amount of 4–5 S RNA (fig. 1a). After 4 hr the proportion between the 38 S and 30 S fractions became roughly 1:1 (fig. 1b). The 30 S and 23 S fractions are likely to be the precursors of the 28 S and 18 S rRNA, respectively [13]. RNA extracted from nucleoli of α-amanitin-treated glands contained principally the same fractions as the control. However, the proportion of the 38 S and 30 S RNA reached in the control after 1 hr of incubation (fig. 1a) was obtained in treated glands only after 4 hr (fig. 1c). Hence, α-amanitin does not inhibit transcription but affects the post-transcriptional process of conversion of pre-ribosomal RNA.

The radioactivity profile of chromosomal RNA from untreated chromosomes I–III (possessing no nucleolus) revealed H RNA and RNA in the range of 4–5 S at all incubation times (1.4 and 16 hr). In addition, after longer incubation times (4 and 16 hr) the chromosomes contained also 30 S and, with great probability, 23 S and 18 S RNA (fig. 2a). The delayed labelling of these fractions indicates again their origin from pre-rRNA. These results are in a good agreement with data of Ringborg et al. [7, 14] and Pelling [15], who isolated the same fractions from the nucleolus-free chromosomes of *Ch. tentans* after long time labelling.

Electrophoretic separations of chromosomal

* In conformity with other authors we use the nominal values of 18, 23, 28, 30 and 38 S in referring to the different rRNA fractions. By use of several marker rRNAs (rat liver, Pelargonium, *E. coli*) the molecular weight of these fractions was determined to be about 0.72, 1.0, 1.4, 1.75 and 2.8 × 10^6, respectively (unpublished results [9]).

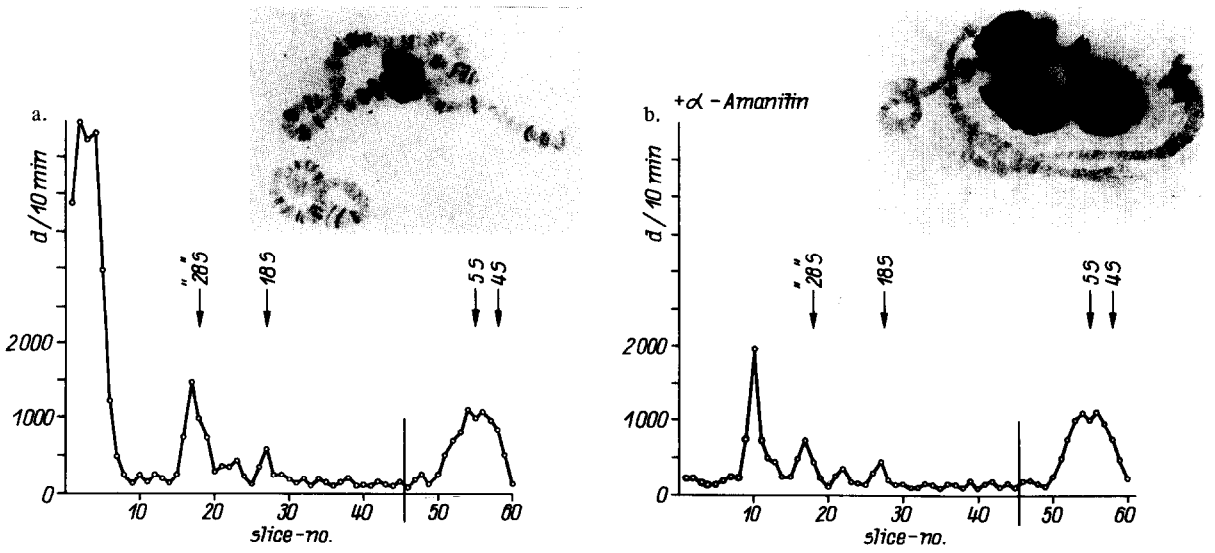

Fig. 2. 4 hr labelling of chromosomal RNA in the absence (a) and presence (b) of 1 μg/ml α-amanitin. For one RNA analysis 70–100 chromosomes I–III were pooled. Preincubation time for the treated glands was 30 min. For further details see fig. 1. Insets: autoradiographs of chromosome sets treated as described above.

RNA after 30 min preincubation of glands with α-amanitin followed by incubation in a radioactive medium in the presence of α-amanitin resulted in a complete or nearly complete (5–10%) absence of H RNA (fig. 2b), whereas without preincubation the labelling of H RNA was suppressed to about 20% of the control. The chromosomes contained only 4–5 S RNA and after long incubation times (4 and 16 hr) 30 S, 23 S and 18 S RNA. In addition to these fractions, a peak at the position of the native ribosomal precursor (38 S) was obtained. In untreated glands this peak was detected only within the nucleolus or, exceptionally, after unfavourable incubation conditions resulting in chromosome damage.

A comparison of cytoplasmic RNA isolated from untreated and treated cells after 16 hr incubation shows that both processing and transport of rRNA and 4–5 S RNA is not prevented by the drug (fig. 3). In contrast to the control H RNA was absent after α-amanitin treatment.

4. Discussion

Our autoradiographic experiments, together with similar results of Beermann [1] and Egyházi et al. [3], demonstrate that inhibition of RNA synthesis by α-amanitin in Chironomus salivary gland cells is restricted to the non-nucleolar regions of the chromosome set. In the experiments of Beermann maximally 80% of the cells of a given gland reacted to 10 μg/ml α-amanitin by suppression of puffing and chromosomal ^{3}H-uridine incorporation. We obtained a complete suppression of puff labelling in all cells at the much lower concentration of 0.5 μg/ml. This difference may be due, at least in part, to the simple phosphate buffered sucrose medium used for *Ch. tentans* [1].

In conformity with the absence of puff labelling H RNA is absent from the RNA profile of chromosomes I–III (fig. 2b), a result likewise obtained for *Ch. tentans* [3]. Under our experimental conditions, however, H RNA forms only a sharp peak near or at the top of the 2.4% acrylamide gel without indications for fractions in the range of 15–45 S, the synthesis of which was found to be less sensitive to α-amanitin by Egyházy et al. [3].

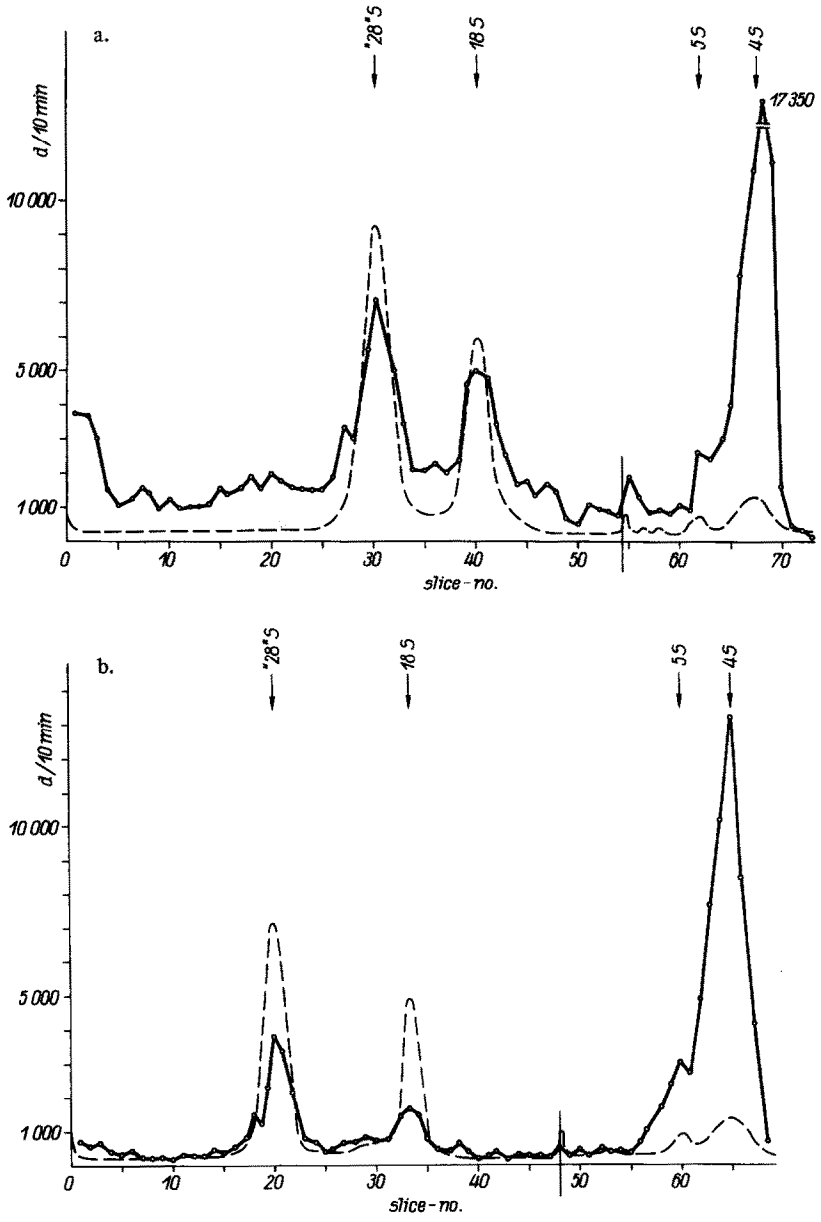

Fig. 3. 16 hr labelling of cytoplasmic RNA in the absence (a) and presence (b) of 1 μg/ml α-amanitin. Preincubation of treated glands was omitted. Cytoplasm was isolated by micromanipulation and RNA extracted as described in Material and methods. (- - - -): absorbance of unlabelled carrier RNA of *Ch. thummi*. For further details see fig. 1.

In contrast to H RNA the synthesis of 4–5 S RNA is not significantly influenced by α-amanitin. Whether this type of RNA contributes to some α-amanitin-resistant puffs (inset of fig. 2b; [1–3]) and/or to the rather high diffuse label in autoradiographs of α-amanitin-treated chromosomes [1–3] remains to be established.

The transcription of nucleolar genes is not im-

paired by α-amanitin but there is evidence for a delayed processing of pre-rRNA. This delay and the appearance of 38 S pre-rRNA on the chromosomes only after α-amanitin-treatment are indications for an additional post-transcriptional effect of the toxin.

The different sensitivity to α-amanitin of the above mentioned types of RNA shows that there must exist in insects, also, different forms of RNA polymerase. As shown by other authors [16] polymerase I transcribing rDNA resides in the nucleolus and at least 2 further forms (II and III) are extracted from the nucleoplasm (i.e. extranucleolar chromatin). Since the genes of 4 S RNA [17] and 5 S RNA [18] as well as polymerase III [16] were localized outside the nucleolus and both the synthesis of the 2 RNA species and the activity of polymerase III remain unaffected by α-amanitin, one may speculate that tRNA genes are transcribed by polymerase III [19]. Whether the sites of tRNA synthesis in polytene chromosomes correspond to α-amanitin-resistant puffs (see p. 149) is questionable. Because α-amanitin prevents the activity of polymerase II [4–6] and, as shown in our experiments, puff labelling and H RNA synthesis, this form of polymerase is concluded to be the predominant polymerase in most of the puffs transcribing H RNA.

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